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No Superoxide Dismutase Activity of Cellular Prion Protein *in vivo*

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Prion diseases are characterized by the deposition of PrP^{Sc}, an abnormal form of the cellular prion protein PrP^{C}, which is encoded by the *Prnp* gene. PrP^{C} is highly expressed on neurons and its function is unknown. Recombinant PrP^{C} was claimed to possess superoxide dismutase (SOD) activity, and it was hypothesized that abrogation of this function may contribute to neurodegeneration in prion diseases. We tested this hypothesis *in vivo* by studying copper/zinc and manganese SOD activity in genetically defined crosses of mice lacking the *Sod1* gene with mice lacking PrP^{C}, and with hemizygous or homozygous *tga20* transgenic mice overexpressing various levels of PrP^{C}. We failed to detect any influence of the *Prnp* genotype and gene dosage on SOD1 or SOD2 activity in heart, spleen, brain, and synaptosome-enriched brain fractions. Control experiments included crosses of mice lacking or overexpressing PrP^{C} with mice overexpressing human Cu^{2+}/Zn^{2+}-superoxide dismutase, and confirmed that SOD enzymatic activity correlated exclusively with the gene dosage of *bona fide* human or murine SOD. We conclude that PrP^{C} *in vivo* does not discernibly contribute to total SOD activity and does not possess an intrinsic dismutase activity.

Key words: Prion disease / PrP^{C} / SOD / Synaptosomes / Transgenic mice.

Introduction

Prion diseases are lethal neurodegenerative disorders that affect humans and a variety of animal species. They include bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in mule deer and elk, and Creutzfeldt-Jakob disease (CJD) in humans. The causative agent is termed prion (Prusiner, 1982) and was proposed to be identical with PrP^{Sc}, a pathological conformer of the cellular protein PrP^{c} encoded by the *Prnp* gene (Oesch *et al.*, 1985; Basler *et al.*, 1986). PrP^{C} is a glycosyl-phosphatidylinositol (GPI)-anchored glycoprotein expressed on the surface of almost all cells in the body, but at particularly high levels on neurons. PrP^{C} has been shown to be essential for the development of prion disease, and $Prnp^{o/o}$ mice, which lack PrP^{C}, are resistant to scrapie (Büeler *et al.*, 1993).

The physiological function of PrP^{C} is unknown, and may be linked to prion pathophysiology. If PrP^{C} was an enzyme, one could speculate that conversion to PrP^{Sc} may trigger changes in its activity or substrate specificity, which – by a loss-of-function or gain-of-function – may conceivably contribute to prion pathology. The latter line of thought gained considerable momentum when it was shown that synthetic peptides encompassing the octarepeat region of PrP^{C} bind copper (Hornshaw *et al.*, 1995) and a PrP^{C}-copper complex was also described *in vivo* (Brown *et al.*, 1997a; Stockel *et al.*, 1998).

PrP^{C} was hypothesized to act as an antioxidant (Brown *et al.*, 1999; Sayre *et al.*, 1999; Wong *et al.*, 1999), since cultured cells lacking PrP^{C} were reported to display an altered response to oxidative stress *in vitro* (Brown *et al.*, 1997b, 1998) and *in vivo* (Klamt *et al.*, 2001; Wong *et al.*, 2001; Brown *et al.*, 2002) and show a higher rate of apoptosis upon growth-factor withdrawal (Kuwahara *et al.*, 1999). It was then reported that $Prnp^{o/o}$ mice exhibit decreased activity of the Cu^{2+}/Zn^{2+}-superoxide dismutase (SOD1; Brown *et al.*, 1997b), a major radical scavenger enzyme located in the cytosol and other subcellular compartments (Kira *et al.*, 2002). If this was correct, PrP^{C} might contribute indirectly to SOD1 activity, or may be an SOD itself (Brown *et al.*, 1999; Wong *et al.*, 2000), whose reduced availability (thanks to conversion into PrP^{Sc}) may reduce scavenging of reactive oxygen species and contribute to neurodegeneration in prion diseases.

The data discussed above may provide a simple and attractive theory of prion pathogenesis, but have been challenged in various ways. Firstly, the results favoring an SOD-like activity of PrP^{C} were mainly generated with recombinant bacterially expressed proteins – which may or may not reproduce the activity of PrP^{C} *in vivo*. Secondly and more worryingly, Waggoner and colleagues found no correlation between brain copper content, level of PrP^{C} expression, and cuproenzyme activity in mice overexpressing or lacking PrP^{C} (2000). Thirdly, it is now established that unintentional overexpression of the PrP^{C}-homologue Dpl in some PrP^{C}-deficient mouse strains and cell lines accounts for at least some of the alterations described above (Moore *et al.*, 1999; Behrens and Aguzzi, 2002).

The contradictory reports on whether PrP^{C} exhibits SOD activity *in vivo* prompted us to analyze this question in defined genetic mouse models. Using two independ-

[a] These authors contributed equally to this study.

ent assays, we were not able to detect any PrP^C-dependent modulation of SOD activity in heart, spleen, liver, brain as well as in synaptosome-enriched brain fractions. We conclude that a direct or indirect contribution of PrP^C to SOD activity in these organs, at least under physiological conditions, appears to be unlikely.

Results and Discussion

The current study was undertaken to test whether PrP^C modulates the total SOD activity in mouse tissues *in vivo*. Formally, PrP^C may alter SOD activity, because (i) it possesses intrinsic dismutase activity, or (ii) because it modifies, directly or indirectly, the activity of the endogenous SOD1 enzyme – for example by altering copper metabolism. Finally (iii), PrP^C may conceivably modify by unknown mechanisms, the activity and/or the tissue distribution of the mitochondrial SOD2 enzyme.

In order to prove or disprove each one of these hypotheses *in vivo*, we determined SOD1 and SOD2 activities in various crosses of mice lacking the *Prnp* and/or *Sod1* genes with mice transgenic for murine *Prnp* and for the human copper-zinc SOD, *hSod1*. Transgenic *Sod1*$^{-/-}$; *Prnp*$^{o/o}$ and *Sod1*$^{-/-}$;*Prnp*$^{o/o}$;*tga20*$^{+/-}$ or *Sod1*$^{-/-}$;*Prnp*$^{o/o}$;*tga20*$^{+/+}$ mice were obtained by intercrossing *Sod*$^{-/-}$ mice (Ho *et al.*, 1998) with *tga20* mice (Fischer *et al.*, 1996) which overexpress PrP^C (Figure 1A). For control experiments, we intercrossed transgenic mice overexpressing the human SOD1 gene, *hSod1* (Epstein *et al.*, 1987), with *tga20* mice. Genotypes were screened by PCR analysis (Figure 1B–E) and confirmed by assessing the presence or absence of the proteins of interest by Western blot (SOD1, MnSOD, PrP^C), as well as functionally by analyzing SOD1 and SOD2 enzyme activity (Figure 2). Double and triple transgenic mice obtained in these crossings did not show obvious phenotypic alterations, except for mice homozygous for a disrupted SOD1 allele that exhibited reduced fertility, as described earlier (Ho *et al.*, 1998). All mice developed normally, had a normal life expectan-

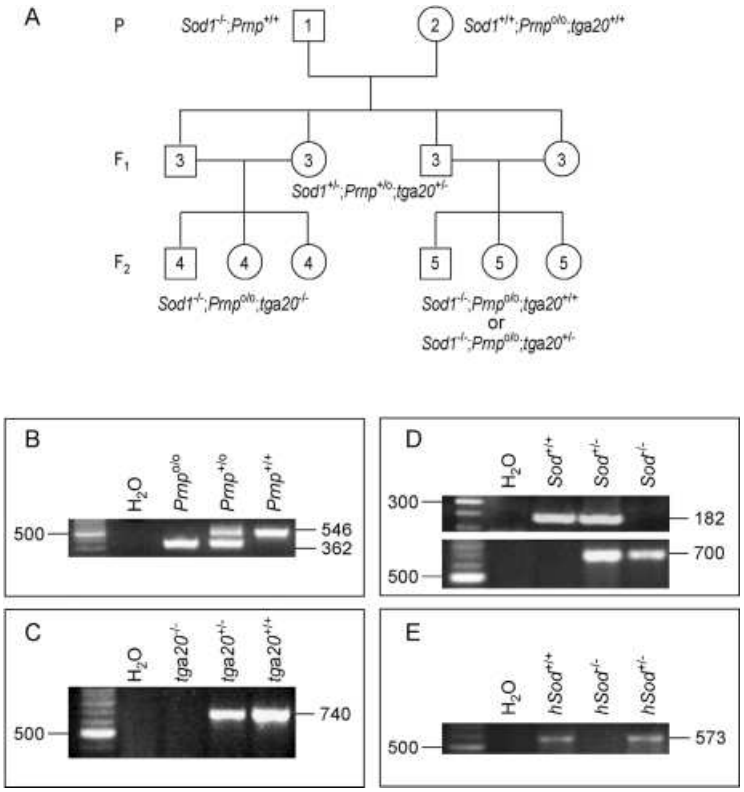

Fig. 1 Breeding Strategy Depicted by a Pedigree (A) and PCR-Based Mouse Genotyping (B-E).
(A) Mice lacking SOD while expressing wild-type levels of PrP^C (*Sod*$^{-/-}$;*Prnp*$^{+/+}$; Ho *et al.*, 1998) were intercrossed with *tga20* mice that transgenically express PrP^C under the control of the *Prnp* promoter on a *Prnp*-deficient background leading to an overexpression of PrP^C in all tissues (Fischer *et al.*, 1996) while displaying wild-type levels of SOD1 (*Sod1*$^{+/+}$;*Prnp*$^{o/o}$;*tga20*$^{+/+}$). The F_1-generation was intercrossed with each other to obtain the indicated genotypes in the F_2-generation. (B) Mouse genotypes were identified by PCR analysis. The *Prnp*-status was revealed by a PCR using three primers within the same reaction (see Materials and Methods), resulting in a 362 bp band for the neo-cassette and a larger 546 bp band for *Prnp*$^+$. (C) Specific primers were used for detecting the *tga20* transgene resulting in a 740 bp band. (D) Presence of the *Sod*$^+$ wild-type allele was confirmed by a 182 bp band which is abolished in SOD1$^{-/-}$-mice. The *Sod*$^-$ allele was demonstrated by an amplicon of 770 bp displaying the exon 4/neo cassette of the targeted *Sod1*-locus, whereas in (E) the human *hSod1* transgene was revealed by a band of 573 bp. (B-E) Respective genotypes of mice are indicated and a 100 bp DNA ladder (Boehringer, Rotkreuz, Switzerland) is shown on the left. Numbers on the right indicate respective size of the PCR amplicons.

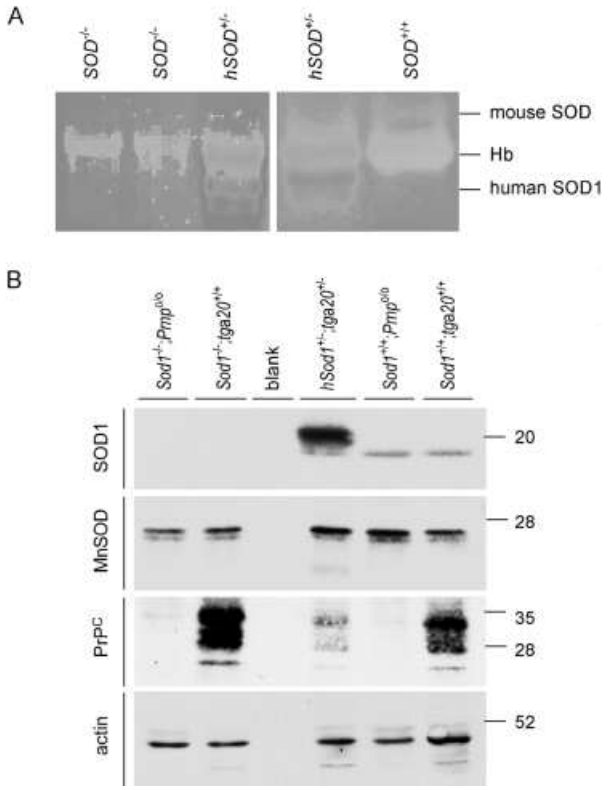

Fig. 2 SOD Activity in Peripheral Blood and Protein Expression in Spleen Homogenates.
(A) A non-denaturing gel-electrophoresis of peripheral blood was performed resulting in mouse/mouse homodimers (mouse SOD) migrating above hemoglobin (Hb), mouse/human heterodimers co-migrating with hemoglobin and a strong band of human homodimeric SOD1 (human SOD1) below hemoglobin in transgenic mice expressing hSOD1. Respective genotypes of mice are indicated. (B) Western blot analysis of spleen homogenates of various mouse genotypes, as indicated, revealing the expression, overexpression or absence of SOD1, MnSOD and PrP^C, as shown on the left. The human SOD1-band migrates slightly slower and exhibits a strong band due to its overexpression in transgenic *hSod1* mice. Equal loading is demonstrated by developing membranes with an anti-β-actin antibody (lower panel). Molecular mass in kDa is indicated on the right.

cy (>350 days at the time of writing) and did not show histological abnormalities in brain, spleen, liver, kidney and heart (data not shown).

Next, we evaluated SOD activity in brain, spleen, liver and heart of mice expressing varying amounts of PrP^C. Using the xanthine/xanthine oxidase formazan based SOD assay, we were not able to detect any difference in total SOD activity between brain homogenates of *Prnp*$^{o/o}$, wild-type, and *tga20* mice which lack, express physiological amounts of, or overexpress PrP^C, respectively (Figure 3A, B). These results are in line with those obtained by Waggoner and colleagues (2000). In addition, we were not able to detect any SOD activity other than SOD2 in mice overexpressing PrP^C but lacking SOD1 (*Sod*$^{-/-}$;*tga20*$^{+/-}$ or *Sod*$^{-/-}$;*tga20*$^{+/+}$; Figure 3A-D). This indicates that PrP^C, even when expressed at supraphysiological levels, does not exhibit any intrinsic SOD activity *in vivo*. Similar results to those in brain (Figure 3A, B) were obtained in heart (Figure 3C, D), spleen (Figure 3E) and liver (data not shown), consistently and regardless of whether samples were analyzed in kinetic (Figure 3A, B, E) or endpoint mode (Figure 3C, D), in which decrease in the reaction speed or inhibition of formazan production are plotted, respectively.

Inhibition of SOD1 activity by KCN revealed no detectable levels of MnSOD in brain (Figure 3B), liver, and spleen of any of the mice investigated (data not shown) and confirmed the specificity of the assay. In contrast, MnSOD was readily detectable in mitochondria-rich heart homogenates and, similar to SOD1, appeared to be independent of PrP^C-expression levels (Fig 3C, D). Multiple independent experiments and use of alternative homogenization buffers and procedures (0.5% NP-40 and 0.5% DOC or sonication followed by ultracentrifugation at 78 000 *g* for 30 min) as described earlier (Ewing and Janero, 1995) produced the same results (data not shown). An additional, independent SOD assay based on auto-oxidation (Nebot *et al.*, 1993) yielded identical results (Figure 3F). Repeated measurements confirmed that the latter assay was robust and highly reproducible with control material and all experimental samples (data not shown).

The highest concentration of PrP^C is found in synaptosome-enriched subcellular fractions, leading to speculations that PrP^C may serve as a copper buffer in the synaptic cleft (Brown *et al.*, 1997a; Kretzschmar *et al.*, 2000). We therefore assayed the SOD activity of synaptosome-enriched brain fractions of mice of all the genotypes described above. In order to confirm the presence of SOD1 and of PrP^C in crude synaptosomal brain fractions, aliquots of each sample were subjected to Western blot analysis in addition to SOD activity assays (Figure 4A). Again, and in flagrant contrast to other reports (Brown and Besinger, 1998; Brown *et al.*, 1999; Milhavet *et al.*, 2000; Wong *et al.*, 2000; Brown *et al.*, 2002; Klamt *et al.*, 2001; Milhavet and Lehmann, 2002) we did not find any contribution of PrP^C to the overall SOD activity, nor did we detect any intrinsic SOD activity of PrP^C in crude synaptosomal brain fractions (Figure 4B).

Our results indicate that modulation of PrP^C levels does not influence the SOD activity in a series of tissues, including brain. The data presented here were collected from organs of genetically defined mouse models lacking or expressing various levels of PrP^C in the presence or absence of endogenous SOD1, whereas most data deposing in favor an SOD activity of PrP^C were recorded with recombinant proteins. In addition, the xanthine-xanthine oxidase based assay (Okado-Matsumoto and Fridovich, 2001) used in this study may be more sensitive and less error-prone than the NBT-method mainly used in previous studies (Brown *et al.*, 1997b, 1999; Brown and Besinger, 1998; Milhavet *et al.*, 2000; Wong *et al.*, 2000). The NBT assay was shown to potentially interfere with xanthine oxidase (Ukeda *et al.*, 1997) and resulted in a

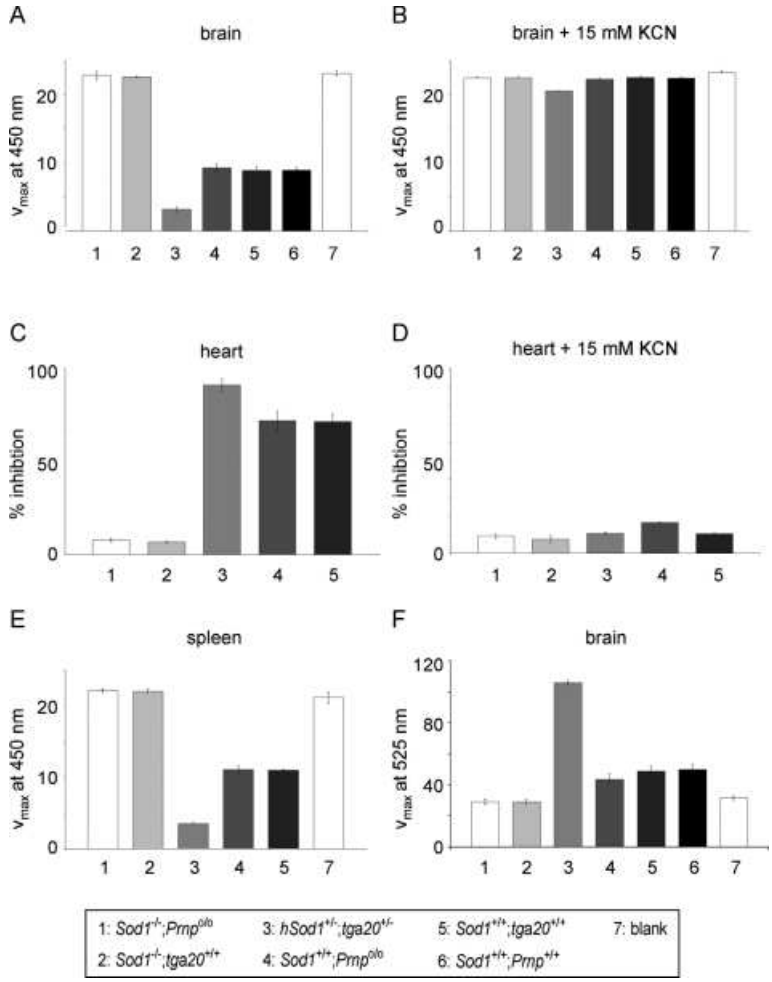

Fig. 3 SOD Activity in Brain, Spleen and Heart Homogenates.
(A) Kinetic measurement of WST-Formazan reduction in a xanthine-xanthine oxidase based SOD-assay at 450 nm over 30 min at 37 °C displaying no significant difference in brain homogenates of mice lacking or overexpressing PrP^C (lanes 4 – 6). No significant SOD-activity was measurable in mice lacking SOD1 (lanes 1 – 2), even when PrP^C was overexpressed (lane 2). As positive control mice overexpressing the human SOD1 (lane 3) were used yielding high SOD levels. (B) KCN-inhibition of same samples as in (A) resulting in a complete inhibition of SOD1, thus displaying MnSOD activity. While the MnSOD enzyme was present in brain extracts (see Figure 2B), its activity even on a *SOD1*$^{-/-}$-background (lanes 1 – 2) could not be detected in brain, spleen (not shown) and liver (not shown) samples. Merely a residual SOD1 activity in transgenic *hSod1* mice was detectable, since 15 mM KCN were not sufficient to completely abolish human SOD1 overexpression. Values are indicated as v_{max} in mOD/min in the linear range of the assay. (C) and (D) indicate percent inhibition of WST-Formazan reduction in a xanthine-xanthine oxidase based SOD-assay with an endpoint measurement at 450 nm in heart homogenates yielding similar results as in (A). In mitochondria-rich heart fractions, MnSOD is readily detectable upon adding 15 mM KCN at similar levels independent of the level of PrP^C expression. (E) Kinetic measurement of WST-formazan reduction in a xanthine-xanthine oxidase based SOD-assay in spleen homogenates also displays no contribution of PrP^C to the SOD-activity (lanes 4 and 5) or an intrinsic SOD-like activity in the absence of SOD1 (lane 2 *vs.* 1). Again, mice overexpressing the human SOD1 (lane 3) served as positive control. (F) Similarly as in the formazan based assay (see 3A), no SOD-like activity of PrP^C in brain homogenates was detectable when a SOD-assay was used that is based on auto-oxidation. (A-F) Six different mouse genotypes are displayed in lanes 1 – 6. Lane 1: *Sod1*$^{-/-}$;*-Prnp*$^{o/o}$, lane 2: *Sod1*$^{-/-}$;*Prnp*$^{o/o}$;*tga20*$^{+/+}$, lane 3: *hSod1*$^{+/-}$;*Prnp*$^{+/o}$;*tga20*$^{+/-}$, lane 4: *Sod1*$^{+/+}$;*Prnp*$^{o/o}$, lane 5: *Sod1*$^{+/+}$;*Prnp*$^{o/o}$;*tga20*$^{+/+}$, lane 6: *Sod1*$^{+/+}$;*Prnp*$^{+/+}$, lane 7: water or buffer (blank). Several independent experiments were performed of which one representative is shown. Experiments were performed in triplicate and mean value ± standard deviation is depicted.

poor reproducibility in our hands. While PrP^C or peptides thereof might exhibit a SOD1-mimetic function under rather artificial settings *in vitro*, our results indicate that *in vivo* a contribution of PrP^C to the SOD activity or a direct SOD-like function of PrP^C, at least under physiological conditions, appears to be unlikely. Certainly, it cannot be excluded that PrP^C merely under conditions of stress exhibits a SOD-like function and, consequently, abrogation of PrP^C, *e.g.* in the course of prion disease, where PrP^C is converted into PrP^{Sc}, might be causally linked to increased free radical formation and thus accelerate neurodegeneration. This scenario, however, appears to be less likely, since SOD1-activity levels and SOD1 protein expression are not decreased in brains of scrapie-infect-

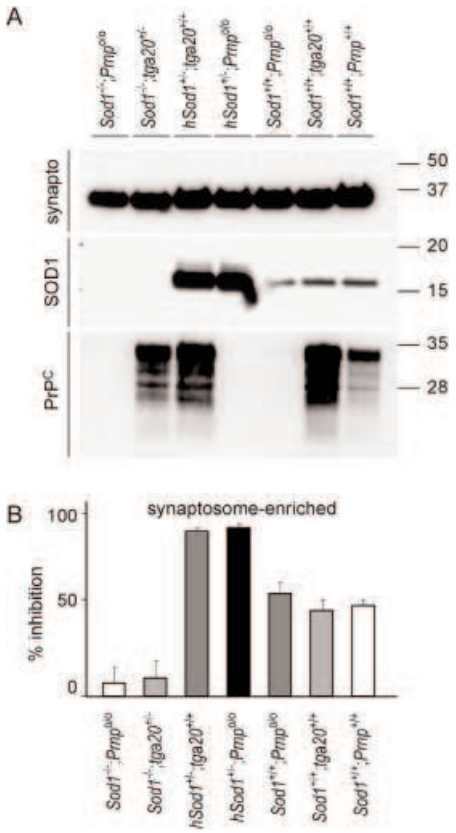

Fig. 4 Protein Expression and SOD Activity in Synaptosome-Enriched Brain Fractions.
(A) Western blot analysis of crude synaptosomal brain fractions of mouse genotypes, as indicated, consisting of synaptophysin-positive material (synapto, upper panel) displaying SOD1- and PrP^C-protein in the respective samples. (B) Same samples as depicted in (A) were subjected to WST-formazan reduction in a xanthine-xanthine oxidase based SOD-assay with an endpoint measurement at 450 nm. Percent inhibition of WST-formazan reduction of crude synaptosomes is illustrated. Again, no contribution of PrP^C to the overall SOD activity (lanes 5 – 7), nor a SOD-like activity of PrP^C, even when overexpressed, was detectable in the absence of endogenous SOD1 (lanes 1 – 2). Synaptosomal fractions of mice overexpressing human SOD1 served as positive controls (lanes 2 – 3). Two independent experiments were performed of which one representative is displayed. Each experiment was performed in triplicate and mean value ± standard deviation is depicted.

ed hamsters (Choi *et al.*, 1998) and mice (Lee *et al.*, 1999). In addition, we were also not able to detect any alteration of SOD1 protein levels in terminally scrapie-sick mouse brains (G.H., F.L.H. and A.A., unpublished observations).

The negative results reported here certainly do not negate a role for reactive oxygen species in prion pathogenesis. Oxidative stress may play a role in a variety of neurodegenerative diseases (Perry *et al.*, 2002). It will therefore be interesting to test whether absence or overexpression of SOD1 alters susceptibility of mice to prion infection, and to assess the capability of mice lacking SOD1 to cope with oxidative stress in the presence or absence of PrP^C.

Materials and Methods

Generation and Genotyping of Transgenic Mice

Prnp$^{o/o}$ (Büeler *et al.*, 1992) and *tga20* mice (Fischer *et al.*, 1996) were intercrossed with *Sod1*$^{-/-}$ mice lacking the endogenous SOD1 protein (Ho *et al.*, 1998; Figure 1A), or with transgenic *hSod1* mice (Epstein *et al.*, 1987) which overexpress the human SOD1 protein. The following genotypes were obtained and used for further analysis: (i) *Sod1*$^{-/-}$;*Prnp*$^{o/o}$, (ii) *Sod1*$^{-/-}$;*Prnp*$^{o/o}$;*tga20*$^{+/-}$ or *Sod1*$^{-/-}$;*Prnp*$^{o/o}$;*tga20*$^{+/+}$, (iii) *hSod*$^{+/-}$;*Prnp*$^{o/o}$, (iv) *hSod*$^{+/-}$;*Prnp*$^{o/o}$;*tga20*$^{+/-}$ or *hSod*$^{+/-}$;*Prnp*$^{o/o}$;*tga20*$^{+/+}$. As controls, the following littermates were used: (v) *Sod1*$^{+/+}$;*Prnp*$^{o/o}$, (vi) *Sod1*$^{+/+}$;*Prnp*$^{+/+}$ and (vii) *Sod1*$^{+/+}$;*Prnp*$^{o/o}$;*tga20*$^{+/+}$.

All genotypes were confirmed by PCR analysis of tail DNA (Figure 1B-D). The following primers were used: Detection of the *Prnp*$^+$ and *Prnp*^{o} alleles using 3 primers within the same PCR reaction as described (Fischer *et al.*, 1996): (5' ATT CGC AGC GCA TCG CCT TCT ATC GCC 3'), (5' GTA CCC ATA ATC AGT GGA ACA AGC CCA GC 3'), (5' CCC TCC CCC AGC CTA GAC CAC GA 3'). Detection of the transgenic *tga20*$^+$ allele: (5' CAA CCG AGC TGA AGC ATT CTG CCT 3'), (5' CCT GGG ACT CCT TCT GGT ACC GGG TGA CGC 3'). Detection of the transgenic *hSod1* allele: (5' TGG GTA TTG TTG GGA GGA GG 3'), (5' TCT GTT CCA CTG AAG CTG TT 3'). Detection of the *Sod*$^+$ allele by amplifying exon 5 of the murine *Sod1*-locus which is removed in the *Sod1*$^{-/-}$ mice by targeted disruption (Ho *et al.*, 1998): (5' CTT GAT CAT TCA AAC CTA AAT GTT CTT 3'), (5' CAG TTG AGT CTG AGA CTT CAG ACC ACA 3'). Detection of the *Sod*$^-$ allele by amplifying the exon 4/neo cassette in *Sod1*$^{-/-}$ mice (Ho *et al.*, 1998): (5' GAA CAT CGT GTG ATC TCA CTC TCA GGA GAG 3'), (5' AAA AGC GCC TCC CCT ACC CGG TAG AAT TGA 3').

Non-Denaturing Gel-Electrophoresis and SOD Zymography

To confirm functionally the presence or absence of endogenous SOD and/or transgenic human Cu^{2+}/Zn^{2+}-superoxide dismutase (hSOD1) activity, erythrocytes were subjected to non-denaturing gel-electrophoresis as described (Beauchamp and Fridovich, 1971). Erythrocytes were lysed, loaded on a non-denaturing gel, and electrophoresed. Gels were stained with 1 mg/ml nitro-blue tetrazolium (NBT) for 20 min and thereafter soaked in 0.036 M potassium phosphate buffer (all reagents were purchased from Sigma Inc., Buchs, Switzerland, unless otherwise specified), pH 7.8, containing 10 µg/ml riboflavin and 3 µl/ml TEMED. Generation of superoxide radicals was induced by subjecting the gels to ultraviolet light resulting in reduction of NBT. Areas with SOD activity remained unstained due to superoxide dismutation (Figure 2A).

Histological Analysis

Mice were deeply anaesthetized, and brain, heart, liver and spleen were removed and fixed in 4% paraformaldehyde in PBS. After embedding the organs in paraffin, tissue was processed according to standard procedures and routinely stained with hematoxylin and eosin (H&E; data not shown).

Preparation of Synaptosome-Enriched Brain Fractions

Synaptosome-enriched brain fractions were prepared according to a protocol provided by Dr. Lloyd Vaughan (Veterinary Pathology, University of Zürich, Zürich, Switzerland). Mice were deeply anaesthetized and brains were removed. Next, brain tissue was cut into small pieces with a razor blade and homogenized by a teflon/glass homogenizer (12 strokes) in 10 volumes of ice-cold buffered sucrose (320 mM sucrose, 4 mM HEPES, pH 7.4) supplemented with a protease inhibitor cocktail (1 tablet per 10 ml

homogenate; Roche, Rotkreuz, Switzerland). Thereafter, homogenates were centrifuged for 10 min at 1100 *g* at 4 °C. Supernatant was transferred to a new tube and centrifuged for 15 min at 9200 *g* at 4 °C for a second time. The pellet was resuspended in 10 ml ice-cold buffered sucrose and centrifuged once more for 15 min at 10 500 *g* at 4 °C. The resulting pellet consisting of crude synaptosomes was then used for Western blot analysis or for SOD assays.

SOD Assays

Mice were deeply anaesthetized and perfused with ice-cold PBS (pH 7.4) in order to remove blood cells. Brain, liver, spleen and heart were excised, snap-frozen in liquid nitrogen, and stored at – 80 °C until further use. Samples were weighed and thawed in ice-cold 50 mM phosphate buffer (pH 7.4) containing a proteinase inhibitor cocktail (1 tablet per 10 ml homogenate; Roche). Samples were homogenized with a Polytron tissue grinder, and crude homogenates were centrifuged at 15 000 *g* for 30 min at 4 °C. Supernatant was then analyzed by a standard BCA assay (Pierce, Rockford, USA) to determine protein concentration. All samples were then adjusted to the same concentration.

Three different SOD assays were used to analyze SOD and SOD-mimetic activity: First, a xanthine/xanthine-oxidase based assay (Dojindo Molecular Technologies) was performed according to the manufacturer's instruction. This assay utilizes the sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, a water-soluble tetrazolium (WST), as a detector of superoxide radical generation (Peskin and Winterbourn, 2000). The assay was standardized by measuring SOD activity of bovine erythrocytes (data not shown). Selective inhibition of SOD1 activity in *Sod1*$^{+/+}$-*Prnp*$^{+/+}$ mice was achieved by adding 15 mM potassium cyanide (KCN) for 30 min on ice, thus allowing to assess the activity of the manganese SOD (MnSOD or SOD2). Lower doses of KCN, in contrast to other reports (Okado-Matsumoto and Fridovich, 2001), did not result in complete inhibition of SOD1 in our experimental series. Two µg of total protein were analyzed per reaction; experiments were run in triplicates and analyzed in a 96-well plate reader (SpectraMax, Molecular Devices Corporation, Sunnyvale, USA). Upon application of the enzyme solution, the linear increase of absorption at 450 nm was monitored constantly over 30 min in kinetic or endpoint mode at 37 °C. Inhibition of formazan production was plotted against corresponding blank controls.

The second SOD assay is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in aqueous alkaline solution, which generates a chromophore with a wavelength of maximal absorbance at 525 nm (Nebot *et al.*, 1993). The assay was performed according to the instructions of the manufacturer (Calbiochem, San Diego, USA). Tissue extracts were prepared as described above, and 250 µl homogenate with a concentration of 5 µg/µl of total protein were subjected to ethanol/chloroform (67.5/32.5 v/v) extraction. Thereafter, 40 µl of supernatant were incubated with the assay reagents as described according to the manufacturer's instructions. Absorption at 525 nm and 37 °C was monitored for 5 min. Increase of absorption per time of different samples in the linear range of the assay was plotted. All kinetic and endpoint data were processed automatically by the SOFTMax Pro software (SpectraMax).

Thirdly, we attempted to establish an NBT-based assay using the non-enzymatic superoxide generator phenazine methosulfate in combination with NADH (Ewing and Janero, 1995). Samples were prepared as described above. Additionally, sonication of the extract and/or treatment with 0.5% DOC and 0.5% NP-40 followed by ultracentrifugation (78 000 *g*, 30 min) was performed. However, we experienced poor reproducibility and variability in repeated measurements of the same probes; therefore this assay was excluded from further analyses.

Western Blots

Aliquots of the same tissue homogenates as used for SOD assays were run on SDS-PAGE gels (5% stacking and 16% resolving) and transferred on nitrocellulose (Schleicher & Schuell, Dassel, Germany) by wet blotting. Membranes were blocked with 5% Top-Block (Juro, Luzern, Switzerland) in Tris-buffered saline-Tween (TBS-T) at room temperature (RT) for 1 hour and, thereafter, incubated for one hour at RT with the primary antibody diluted in 1% Top-Block in TBS-T. As primary antibody we used an anti-PrP antibody (ICSM18; kind gift of Dr. S. Hawke, London, UK; dilution 1:30 000), an anti-SOD1 antibody (Calbiochem; dilution 1:1 000: approximately 16 kDa), an anti-SOD2 antibody (Stressgen, Victoria, Canada; dilution 1:5 000; approx. 25 KDa) or an anti-β-actin antibody (Jackson, USA; dilution 1: 5 000). Membranes were washed three times for 15 min in TBS-T, and incubated for further 45 minutes with a horseradish peroxidase (HRP)-labeled secondary antibody diluted in 1% Top-Block in TBS-T. As secondary antibody we used rabbit-anti-mouse IgG_1-HRP (Zymed, South San Francisco, USA, dilution 1:10 000) for detecting anti-PrP and anti-β-actin antibodies or goat-anti-sheep IgG-HRP for detecting anti-SOD1 and anti-SOD2 antibodies (Jackson; dilution 1:10 000). Membranes were washed three times for 15 min in TBS-T and developed using enhanced chemiluminescence (ECL, Pierce) detection reagents.

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